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"Removal of nitrate and nitrite by hemodialysis in end-stage renal disease and by sustained low-efficiency dialysis in acute kidney injury"

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ABSTRACT

Background & purpose: It is well established that end-stage renal disease (ESRD) is associated with increased cardiovascular morbidity and mortality both in the adult and pediatric population. Although the underlying molecular mechanisms are poorly understood, compromised nitric oxide (NO) bioactivity has been suggested as a contributing factor. With this in mind, we investigated the effects of hemodialysis on NO homeostasis and bioactivity in blood.

Methods & results: Plasma and dialysate samples were obtained before and after hemodialysis sessions from adults (n = 33) and pediatric patients (n = 10) with ESRD on chronic renal replacement therapy, and from critically ill adults with acute kidney injury (n = 12) at their first sustained low-efficiency dialysis session. Levels of nitrate, nitrite, cyclic guanosine monophosphate (cGMP) and amino acids relevant for NO homeostasis were analyzed. We consistently found that nitrate and cGMP levels in plasma were significantly reduced after hemodialysis, whereas post-dialysis nitrite and amino acids coupled to NO synthase activity (*i.e.*, arginine and citrulline) were only significantly reduced in adults with ESRD. The amount of excreted nitrate and nitrite during dialysis were similar to daily endogenous levels that would be expected from endothelial NO synthase activity.

Conclusions: Our results show that hemodialysis significantly reduces circulating levels of nitrate and cGMP, indicating that this medical procedure may impair NO synthesis and potentially NO signaling pathways.

1. Introduction

Despite significant improvements in the quality and efficacy of hemodialysis therapy, cardiovascular disease remains the leading cause of death for end-stage renal disease (ESRD) in children and adults undergoing dialysis [1,2].

Endothelial dysfunction is a major contributing factor in the pathophysiological continuum of cardiovascular disease in ESRD patients [3]. Increased arterial stiffness and reduced flow-mediated dilatation have been observed in adult hemodialysis patients compared to healthy individuals [4–6], and is associated with an independent increase in cardiovascular risk in this group of patients [3]. Similarly, arterial stiffness was found to be increased in pediatric patients undergoing dialysis compared to healthy children [7,8]. Although the pathogenesis of endothelial dysfunction in ESRD patients is multifactorial [3], impaired availability of nitric oxide (NO) is a well-recognized cause of vascular endothelial dysfunction in hemodialysis patients [9]. In line with this, we have recently observed that a reduction in NO bioavailability in infants on chronic peritoneal dialysis may impair the autoregulation of cerebral blood flow, resulting in an increased risk for cerebral ischemia [10,11]. Previous studies conducted in adults with ESRD on chronic hemodialysis showed a significant reduction in both plasma and salivary levels of nitrate and nitrite after dialysis [12]. Since nitrate and nitrite are metabolites of endogenous NO generation that may be recycled to bioactive NO [13–16], the authors speculated that impaired NO availability may account for the disproportionate high prevalence of cardiovascular-related complications observed in adults with ESRD. However, despite this evidence, the pathways of NO synthesis and signaling have not been fully investigated in ESRD patients.

The main objective of this study was to follow the circulating levels of nitrate and nitrite in adults and children with ESRD undergoing

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routine hemodialysis as well as in critically ill adults with acute kidney injury. In addition, our secondary objectives were to calculate nitrate and nitrite clearances and to examine the plasma levels of cyclic guanosine monophosphate (cGMP), a surrogate marker of biologically active NO signaling as well as amino acids relevant for NO homeostasis before and after a hemodialysis session.

2. Material and methods

2.1. Study population

This study was conducted in Hospital Italiano de Buenos Aires, Argentina, from 8th January 2018 to 24th July 2018. During this period, the number of adults and children with ESRD on regular thrice-weekly hemodialysis was approximately 160 and 16 patients, respectively. In addition, there were approximately 8 adult patients with severe acute kidney injury that initiated slow extended renal replacement treatment every month.

Since the enrolment of ESRD participants was limited to the shift when our dedicated study team was working, there were patients that were not asked to participate in the study. Out of 68 potential eligible participants, our team excluded those patients that were hepatitis C virus-positive (n = 2), had been hospitalized due to metabolic or infectious disease in the month preceding enrolment (n = 10) or had vascular access dysfunction (n = 10). Forty-six patients were asked to participate and one declined participation. Out of 45 patients that accepted participation, 2 were excluded due to incomplete laboratory data.

Our study population consisted of 33 adults with ESRD (19 men, 14 women) on conventional hemodialysis (n = 16) or post-dilution hemodiafiltration (n = 17) and 10 ESRD children (7 male, 3 female) on conventional hemodialysis (n = 5) or post-dilution hemodiafiltration (n = 5), respectively. All these patients were examined at their first dialysis session of the week, which represents the larger interval between consecutive dialysis sessions. They were on their usual dietary intake and baseline blood values were non-fasting samples. In addition, 12 critically ill adults with severe acute kidney injury (8 men, 4 women) were examined on their first sustained low-efficiency dialysis (SLED) session.

This cross-sectional observational study of prospectively enrolled patients described here has been carried out in accordance with The Principle of Ethics of the World Medical Association that originated in the Declaration of Helsinki for experiments involving humans, and that the data are credible. Approval for this study was obtained from the Hospital Italiano de Buenos Aires Ethics Committee for human investigations (Protocol number 3370/2017). Adult patients received oral and written information, and all signed an informed consent form except for 9 critically ill adults who were on mechanical ventilation (n = 7) or unconscious (n = 2) at the time of enrolment and in whom information on the study as well as the signature of the consent was done by the patient's nearest relative(s). In children, informed written consent was obtained voluntary from all parents.

2.2. Data collection

The characteristics of the participants included, etiology of primary renal disease, weight, height, body mass index, current medication, blood pressure, and comorbidities.

Pre- and post-dialysis weights were recorded in all ESRD participants whereas in critically ill adults with severe acute kidney injury the nearest available weight before the onset of their first SLED session was documented. In children, height and post-dialysis weight standard deviation scores (SDS) were calculated by the LMS method using published reference values for healthy Argentinian children [17]. Indirect, non-invasive oscillometric systolic and diastolic blood pressure measurements were recorded pre- and post-dialysis in all participants by trained staff members. The etiology of primary renal disease in adults with ESRD was diabetic nephropathy (n = 7), glomerulopathies (n = 7), anomalies of the kidney and urinary tract (n = 7), hypertensive nephropathy (n = 4), polycystic kidney disease (n = 4), hemolytic uremic syndrome (n = 2), amyloidosis (n = 1), and unknow etiology (n = 1). In children with ESRD the etiologies were congenital anomalies of the kidney and urinary tract (n = 5), glomerulopathies (n = 3), polycystic kidney disease (n = 1), and hemolytic uremic syndrome (n = 1).

The causes of admission to the intensive care unit in critically ill adults who underwent SLED were uncompensated heart failure (n = 3), major surgery (n = 3), sepsis (n = 2), liver failure (n = 1), acute myeloid leukemia (n = 1), pulmonary-renal syndrome (n = 1), and respiratory insufficiency (n = 1). The indication for initiation of acute renal replacement therapy was azotemia (n = 9), volume overload (n = 2), and hyperkalemia (n = 1), respectively.

The characteristics of all the participants are detailed in Supplemental Table S1.

2.3. Hemodialysis procedures

The type of the hemodialysis, *i.e.*, conventional hemodialysis or post-dilution hemodiafiltration as well as the duration of the dialysis session were not modified for the study. All participants were dialyzed thrice-weekly with either a Fresenius 4008 or a Fresenius 5008 dialysis machine (Fresenius Medical Care, Bad Homburg, Germany) using either Fresenius Polysulfone®- or Helixone®-based dialysis membrane (Fresenius S.E., Bad-Homburg, Germany). Both conventional hemodialysis and post-dilution hemodiafiltration were performed with ultrapure bicarbonate dialysate. The prescribed extracorporeal blood flow rate was above 150 mL/min in children and 300 mL/min in adults whereas the prescribed dialysate flow rate was set at 500 mL/min. The delivered dialysis dose was determined by the calculation of Kt/V_{urea} [18]. This index of the adequacy of the dialysis treatment indicates the blood volume that has been completely cleared of urea during a specified dialysis. A $Kt/V_{urea} \geq 1.2$ is the recommended minimum dose per session [18]. In the present study, the measurement of delivered Kt/V_{urea} was determined automatically at the end of the dialysis by the Online Clearance Monitoring Method [19]. The characteristics of the chronic intermittent hemodialysis in ESRD participants is detailed in Supplemental Table S2.

The Genius® dialysis system (Fresenius Medical Care, Bad Homburg, Germany) was used as a daily SLED in critically ill adults with severe acute kidney injury [20]. This system is a mobile and simple to handle dialysis machine that has been designed as a single-pass closed-loop batch hemodialysis system as described elsewhere in detail [21,22]. Briefly, a 90-Liters tank air-free glass container is filled with the dialysate, which consists of pre-packed bicarbonate and electrolyte concentrates diluted in ultra-pure water. The fresh dialysate is taken out of the top compartment of the tank whereas the spent dialysate is returned to the bottom compartment of the tank [21,22]. As in conventional hemodialysis, the effective extracorporeal blood flow can be chosen stepwise, being the dialysis efficiency largely dependent on the blood flow rate. Therefore, the effective extracorporeal blood flow is an important determinant of the overall dialysis time. The characteristics of SLED in critically ill adults is detailed in Supplemental Table S3.

2.4. Collection and analyses of plasma and dialysate

In all participants with ESRD, a blood sample was taken from the inlet blood line to the filter immediately before the commencement of hemodialysis session, as a representative sample of patient's baseline blood values (P0). Approximately 10 min before the end of the hemodialysis session, another blood sample was collected from the inlet blood line (P1), as a representative sample of patient's new steady-state of nitrate and nitrite levels in plasma after completion of hemodialysis.

At the end of the hemodialysis session, an aliquot (5 mL) derived from a small representative sample of the total spent dialysate (including the ultrafiltrate and in the case of post-dilution hemodiafiltration, the substitution volume during the dialysis session) was obtained to measure nitrate and nitrite concentrations (D1). To secure a representative sample of the total spent dialysate, we used the partial spent dialysate collecting method as previously described by Ing et al. [23].

In critically ill adults receiving SLED blood samples were taken at their first dialysis session from the inlet blood line before the onset of dialysis (P0), at approximately 10 min before the end of dialysis (P1) as well as before the start of the second dialysis session, which began on the day after their first session (P2). At the completion of participants' first hemodialysis session, an aliquot (5 mL) derived from a small fraction of the total spent dialysate (including the ultrafiltrate) were obtained to measure nitrate and nitrite concentrations, which were collected by applying the same method as described in ESRD participants (D1) [23].

In addition, samples of fresh dialysate were taken before its entry to the dialyzer in 16 ESRD patients before the commencement of their dialysis session and analyzed for nitrate and nitrite (D0).

In all participants, the amount of blood that was drawn at each time point consisted of approximately 2 mL with EDTA (5 mmol/L). The blood samples were immediately centrifuged at 4700 *g* (5 min, 4 °C), and the collected plasma (about 1 mL) as well as dialysate (5 mL) samples were instantly frozen and stored (−80 °C) for later analyses.

Methods for analyzing nitrate, nitrite and NO signaling, as well as different amino acids, are described below.

Nitrate and nitrite: Similar to that previously described [24,25], a dedicated HPLC system (ENO-20) and auto-sampler (840, EiCom, Kyoto, Japan) was used to measure nitrate and nitrite levels in plasma and dialysate samples. Briefly, the samples were extracted using methanol (1:2) and then centrifuged at 10000 *g* (10 min, 4 °C). Nitrate and nitrite were separated by reverse phase/ion exchange chromatography followed by nitrate reduction to nitrite by cadmium and reduced copper. The nitrite was then derivatized using Griess reagent to form diazo compounds and analyzed by detection at 540 nm.

cGMP: Plasma for cGMP measurements was collected in IBMX-containing tubes (10 μM). Samples were analyzed using cGMP ELISA kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions.

Amino Acids: Urea cycle amino acids and methyl-arginine were analyzed in both plasma and dialysate, as previously described [26,27]. Briefly, after thawing samples on ice, 25 μl of plasma or dialysis solution were crashed with 225 μl of 0.2% formic acid in isopropanol containing the internal standard (1 μmol/L of N_4-Arginine).

Urea cycle amino acid analyses were performed on an ACQUITY UPLC System from Waters Corporation (Milford, MA, USA) coupled to a Waters Xevo® TQ-S triple quadrupole system equipped with an Electrospray Ion Source as previously described [28]. Separation was carried out on a SeQuant® ZIC®-HILIC (100 × 2.1 mm, 3.5 μm, 100 Å) column equipped with a SeQuant® ZIC®-HILIC guard column (20 × 2.1 mm), both from Merck. Mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The elution gradient used was as follows: 0.0, 95% B; time range 0.0–0.5 min, 95% → 80% B; time range 0.5–6.0 min, 80% → 40% B; time range 6.0–8.0 min, 40% → 20% B; time range 8.0–9.5 min, 20% B; time range 9.5–9.7 min, 25% → 95% B and 9.7–12.2 min, 95% B (isocratic column conditioning). The flow rate was set at 300 μl min^{-1}, the injection volume was 3 μl and the column oven was maintained at 27 °C. Detection was performed in positive ionization mode. Quantifier and qualifier ions were as follows: Ornithine (133 → 70/133 → 116), Arginine (175 → 70/175 → 60), Citrulline (176 → 70/176 → 113), N^G-monomethyl-L-arginine (MNMA) (189 → 70), symmetric dimethylarginine (SDMA) (203 → 172/203 → 158), asymmetric dimethylarginine (ADMA) (203 → 46/203 → 158) and N_4-Arginine (179 → 71). For additional confirmation, the ion ratios between the quantifier and qualifier ions for urea cycle aminoacids (maximum deviation = 15%) were calculated.

2.5. Nitrate and nitrite clearance calculation

Nitrate and nitrite clearances were calculated from the measured total effluent volume (TEV) during the treatment time and the effluent to plasma nitrate/nitrite ratio using the following formula:

$$K\ (\text{mL/kg/min}) = TEV\ (\text{mL/min}) * (\text{D1} / \text{P0}) / BW\ (\text{kg}),$$

where *K* is the measured nitrate/nitrite clearance; *TEV* is the total effluent volume adjusted to time as mL per minute, which includes the total spent dialysate, ultrafiltrate, and in the case of post-dilution hemodiafiltration, the substitution volume during the treatment time; D1 is the concentration of nitrate and nitrite in the *TEV* at the end of the dialysis session; P0 is the concentration of nitrate and nitrite obtained from the patient at the beginning of the dialysis session; *BW* represents patient's body weight at the end of the dialysis session in ESRD patients and the nearest available body weight before the start of SLED in critically ill adults with severe acute kidney injury.

The dialysis clearance provides an estimate of the rate of elimination of nitrate and nitrite from the central or plasma compartment by the hemodialysis and the SLED procedures, respectively. The amount of excreted nitrate and nitrite during the dialysis session was calculated by multiplying the delta concertation of these anions, *i.e.*, D1 - D0, and the volume of *TVE* at the end of the hemodialysis as well as SLED session.

2.6. Statistical methods

Comparison of two groups was calculated by Student's paired or unpaired nonparametric *t*-test and Pearson correlation coefficient was used to measure association between two variables. More than two groups were compared by Friedman test together with Dunn's multiple comparisons test. All statistical calculations were made using GraphPad Prism (version 7.0d, La Jolla, CA, USA). Data in all figures are presented as mean ± SEM. All continuous variables in Tables are presented as medians and interquartile ranges (IQR), unless otherwise stated. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Effect of hemodialysis on plasma nitrate and nitrite levels

In adults with ESRD there was a significant reduction in both nitrate and nitrite before the end of their dialysis sessions (Fig. 1, panel A and B). Plasma nitrate was reduced from 67.7 (44.5–90.5) μmol/L to 14.9 (13.3–22.6 μmol/L (78% decrease from baseline), and plasma nitrite was reduced from 0.556 (0.331–0.97) μmol/L to 0.406 (0.262–0.809) μmol/L (27% decrease from baseline), respectively. Concomitantly, there was a significant increase in the concentrations of these anions in the spent dialysate (Fig. 2, panel A and B). Similarly, there was a significant reduction in plasma nitrate before the end of dialysis sessions in children with ESRD, from 153.7 (67.6–225.4) μmol/L to 31.2 (15.7–43.9) μmol/L (80% decrease from baseline). There was however no significant difference in plasma nitrite before the start and the end of their dialysis sessions ($P = 0.94$); Fig. 1, panel D). A significant increase in the concentrations of nitrate and nitrite in the spent dialysate was also observed in children with ESRD (Fig. 2, panel A and B).

The median (IQR) of nitrate and nitrite clearances were 1.1 (0.75–1.86) mL/kg/min and 3.21 (1.61–5.1) mL/kg/min, in adults with ESRD and 1.31 (1.18–1.84) mL/kg/min and 6.63 (4.99–9.91) mL/kg/min, in children with ESRD, respectively.

In adults with ESRD, the median (IQR) of nitrate clearance was 1.078 (0.798–1.761) mL/kg/min with conventional hemodialysis and 0.996 (0.686–2.001) mL/kg/min with post-dilution hemodiafiltration

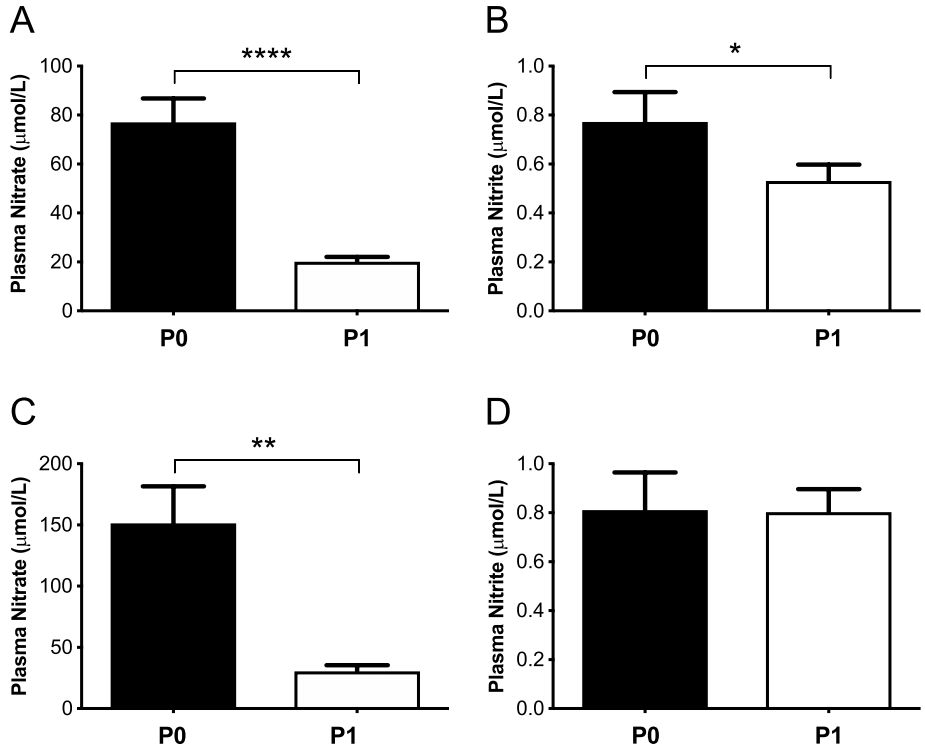

Fig. 1. Plasma levels of nitrate and nitrite in adult (panel A and B) and pediatric (panel C and D) patients with end-stage renal disease before the commencement (P0) and at the end of the hemodialysis session (P1). Statistical significance level:*, < 0.05; ** < 0.01; ****, *< 0.0001.*

(P = 0.75), whereas in children with ESRD nitrate clearance was 1.546 (1.211–2.09) mL/kg/min with conventional hemodialysis and 1.238 (1.157–2.236) mL/kg/min with post-dilution hemodiafiltration (P = 0.54), respectively.

Also, in both adults and children with ESRD we did not observe a significant difference in nitrite clearance between conventional hemodialysis and post-dilution hemodiafiltration; 2.329 (1.636–3.96) mL/kg/min with conventional hemodialysis and 3.762 (1.517–8.03) mL/kg/min with post-dilution hemodiafiltration (P = 0.3) in adults with ESRD, and 6.703 (5.191–10.45) mL/kg/min with conventional hemodialysis and 6.562 (4.818–8.752) mL/kg/min with post-dilution hemodiafiltration (P = 0.69) in ESRD children, respectively.

The amount of excreted nitrate and nitrite during dialysis were 790 (283–1307) and 25.71 (5.70–35.87) μmoles in adults, and 1387 (50.50–2508) and 36.26 (24.57–45.90) μmoles, in children, respectively (Fig. 2, panel C and D).

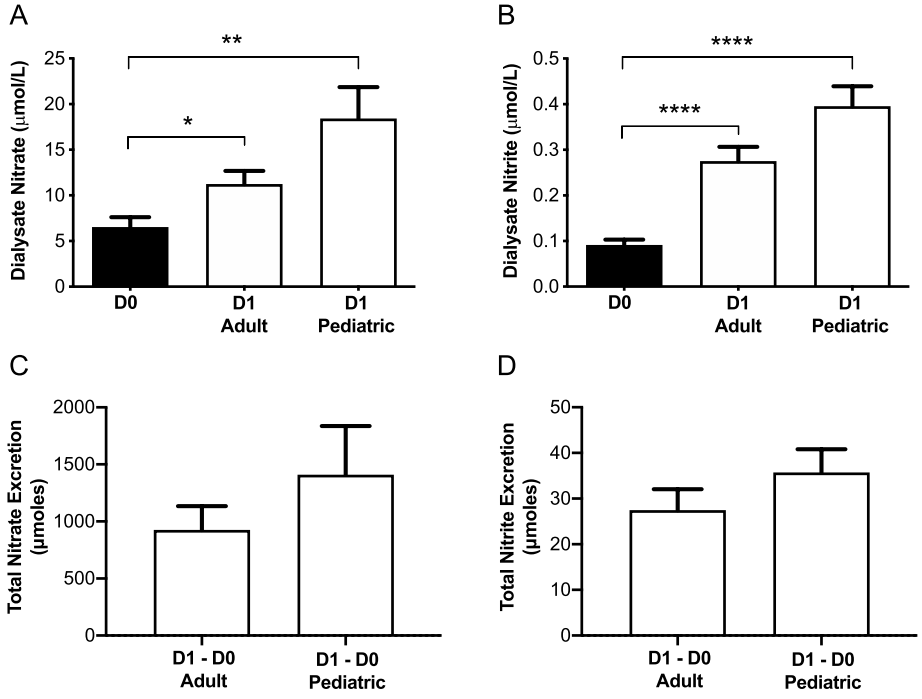

Fig. 2. Levels of nitrate (panel A) and nitrite (panel B) in fresh dialysate (D0) and from a representative sample of spent dialysate at the end of the hemodialysis session (D1) in adult and pediatric patients with end-stage renal disease. Total amount of excreted nitrate (C) and nitrite (D) during the hemodialysis session. Statistical significance level: *, < 0.05; **, < 0.01; ****, < 0.0001.

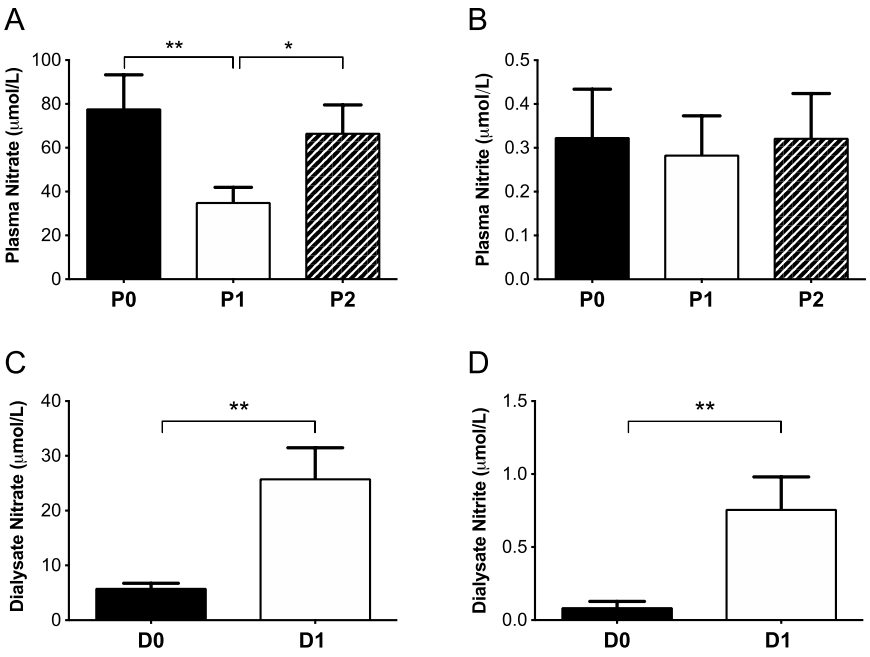

Fig. 3. Levels of nitrate (panel A) and nitrite (panel B) in plasma before the commencement (P0), at the end (P1), and at the start of the second slow extended renal replacement treatment session (P2) as well as nitrate (panel C) and nitrite (panel D) levels in fresh dialysate (D0) and from a representative sample of spent dialysate at the end of the first sustained low-efficiency dialysis session (D1) in critically ill adults with acute kidney injury. Statistical significance level: *, < 0.05; **, < 0.01.

3.2. Effect of sustained low-efficiency dialysis on plasma nitrate and nitrite levels

In critically ill adults with acute kidney injury undergoing their first SLED session, we found that plasma nitrate was reduced from 52.42 (45.97–110.2) μmol/L to 29.95 (15.86–52.6) μmol/L (43% decrease from baseline), whereas no significant changes were observed for plasma nitrite (Fig. 3, panel A and B). Concomitantly, there was a significant increase in the concentrations of these anions in the spent dialysate (Fig. 3, panel C and D).

As depicted in Fig. 3, panel A and B, plasma nitrate and nitrite levels increased back to baseline levels before the commencement of the second SLED session, suggesting a complete recovery in plasma concentration of these anions after dialysis treatment.

The median (IQR) clearance of nitrate and nitrite were 0.683 (0.52–0.834) mL/kg/min and 3.482 (0.85–9.674) mL/kg/min, respectively.

The amount of excreted nitrate and nitrite during SLED were 748 (378–1976) and 10.73 (9.46–93.04) μmoles, respectively.

3.3. Effect of hemodialysis and of sustained low-efficiency dialysis on cGMP and amino acids relevant for NO homeostasis

A significant reduction in the levels of cGMP, a downstream product of NO pathway that also serves as a surrogate marker of biologically active NO signaling [29], was observed before the end of dialysis as compared to baseline values in all treatment groups (Fig. 4, panel A, B, and C).

We also investigated whether dialysis-mediated reduction in the concentration of plasma nitrate, nitrite, and cGMP may have been linked to weakened NOS function, due to limitation of the substrate L-arginine [13]. A significant decrease in plasma levels of arginine, citrulline, and ornithine at the end of dialysis was only observed in adults with ESRD (Fig. 5, panel A). A significant decrease in post-dialysis plasma citrulline-to-arginine ratios, a surrogate measure of endothelial NOS activity, as well as in plasma ornithine-to-citrulline ratios, a surrogate of arginase activity, were also exclusively observed in adults with ESRD (Fig. 5, panel A).

3.4. Pre- and post-dialysis body weight and blood pressure

Chronic hemodialysis was associated with significant differences between pre- and post-dialysis weight in both children 34.7 (29.3–43.1) *vs.* 33.7 (28.5–42.5) kg, $P = 0.04$ and in adults 75.7 (59.6–88.2) *vs.* 73.9 (57.2–85.7) kg, $P < 0.0001$, respectively. This is supported by regression analysis of the pooled adult and pediatric ESRD data showing a positive correlation between delta body weight and ultrafiltration ($r^2 = 0.923$, $P < 0.0001$).

Post-dialysis blood pressure levels tended to be lower as compared to pre-dialysis values; adults: systolic blood pressure 135 (119–142) *vs.* 122 (108–143) mmHg, $P = 0.11$, and diastolic blood pressure 67 (57–79) *vs.* 67 (55–75) mmHg, $P = 0.55$, children: systolic blood pressure 116 (102–123) *vs.* 111 (103–122) mmHg, $P = 0.3$, and diastolic blood pressure 57 (53–69) *vs.* 61 (56–70) mmHg, $P = 0.88$,

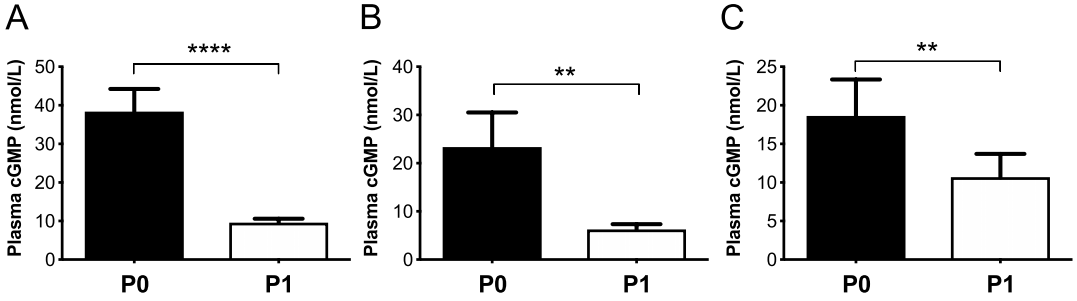

Fig. 4. Plasma cGMP levels in adults (panel A) and in pediatric (panel B) patients with end-stage renal disease and in critically ill adults with acute kidney injury (panel C) before the commencement (P0) and at the end of their dialysis sessions (P1). Statistical significance level: **, < 0.01; ****, < 0.0001.

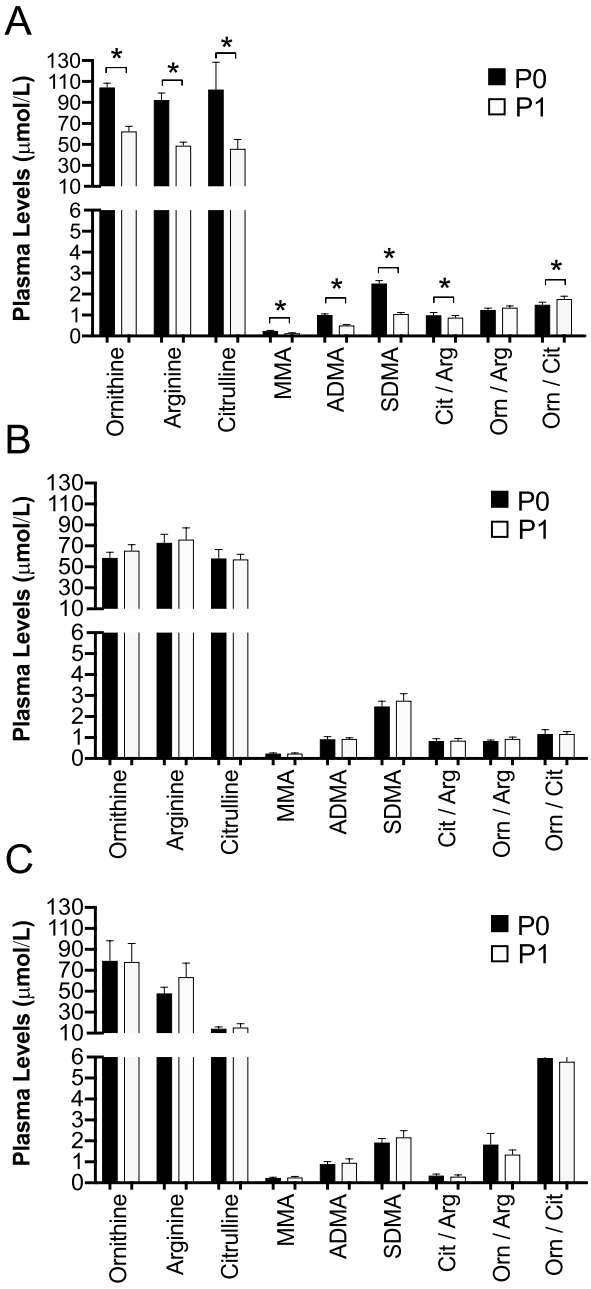

Fig. 5. Plasma levels of amino acids related to nitric oxide bioactivity in adults (panel A) and in pediatric (panel B) patients with end-stage renal disease and in critically ill adults with acute kidney injury (panel C) before the commencement (P0) and at the end of their dialysis sessions (P1). Statistical significance level: *, < 0.05.

respectively.

In critically ill adults with severe acute kidney injury treated with SLED we did not observe a significant difference in systolic or in diastolic blood pressure levels before and after dialysis; 106 (98–135) *vs.* 114 (97–126) mmHg, $P = 0.68$, and 59 (52–72) *vs.* 65 (59–74) mmHg, $P = 0.71$, respectively.

4. Discussion

In the present study, we observed that circulating plasma levels of nitrate and cGMP were invariably reduced after a dialysis session in all groups of patients analyzed. However, plasma nitrite was only reduced in adults with ESRD. Since nitrate is a major circulating pool for subsequent NO bioactivity in our bodies, these findings may suggest an impairment of NO bioactivity and potentially of NO mediated signaling. Considering the important role of NO in maintaining vascular homeostasis [30], the marked dialysis-induced fluctuations in potential NO signaling noted in our study may contribute to the increased cardiovascular risk observed in ESRD patients on chronic hemodialysis and could also shed light on new therapeutic possibilities.

Circulating nitrate and nitrite anions are stable metabolites of NO oxidation, and the sum of these anion concentrations (NOx) has often been used as an index of NOS activity. Accumulating evidence also show that via enterosalivary circulation, nitrate can be converted back to nitrite by commensal bacteria in the mouth and then back again to NO as well as other bioactive nitrogen oxide species via different enzymatic systems in tissues and in the circulation [31]. In addition to NOS-derived NO our daily diet, especially green leafy vegetables, significantly contributes to the total body pool of circulating nitrate and nitrite. Dietary supplementation with nitrate to boost this nitrate-nitrite-NO pathway has been associated with favorable reno-cardio-metabolic effects in preclinical studies [32–34], and has been shown to improve endothelial function and to lower blood pressure in both healthy human subjects [35] and in hypertensive patients [36,37]. The organs involved and the underlying mechanisms contributing to the favorable effects of nitrate, including blood pressure reduction, are likely multifactorial involving reduction of oxidative stress and increased NO bioactivity as well as modulation of mitochondria and immune cell function [15,38,39]. Of note, many of the favorable effects following boosting the nitrate-nitrite-NO pathway have particularly been observed in pathological conditions when the endogenous NOS system is compromised (*i.e.* endothelial dysfunction, oxidative stress).

Currently, there is little knowledge regarding the cardiorenal effects of nitrate treatment in patients with kidney disease. In healthy subjects, short-term treatment, *i.e.*, 1 week, with nitrate or nitrite showed either no effect or an increase of GFR [40–42]. In patients with chronic kidney disease, acute dietary nitrate supplementation (300 mg) was associated with a reduction in blood pressure and in renal resistive index [43].

In the current study we show that circulating levels of NO metabolites and markers of NO signaling were significantly reduced during the dialysis sessions in both adults and children with ESRD on chronic hemodialysis and in critically ill adults with acute kidney injury during their first SLED. In our adult patients with ESRD, nitrate, nitrite, cGMP, arginine, citrulline, and endogenous NOS inhibitors ADMA and SDMA showed similar alterations after dialysis, comparable to previous investigations in adults on chronic hemodialysis and peritoneal dialysis, indicating decreased NO production [12,44–47]. In contrast to the robust and consistent reduction of nitrate and cGMP observed in all our study groups, plasma nitrite was only significantly changed in adults with ESRD. Since uncertainty as to whether the filter itself may have been an additional source of nitrite we infused through a commercial hemodialysis arterial line an unused representative filter with 0.9% saline solution (154 mmol/L sodium, 154 mmol/L chloride) to test this conjecture. Paired samples from both the inlet and from outlet line were obtained at the start of infusion, 0.5, 1, 2, 3, and 4 h, respectively. We were unable to demonstrate any significant differences between pre- and post-filter nitrite levels (data not shown). Aging is commonly associated with endothelial dysfunction, and both chronic kidney disease and ESRD have been associated with premature aging of the microcirculation [48]. Of note, a weakened NOS function was only observed in adult patients with ESRD as suggested by a reduction of the substrate L-arginine and an increase in endogenous NOS inhibitors ADMA and SDMA levels after dialysis. Studies conducted in children with ESRD have showed a positive association between time on dialysis and deteriorating measures of vascular function [49]. Taken together, we speculate that the shorter dialysis vintage in our pediatric study population with ESRD, as compared with adult patients (Table S2), may have contributed to a better NOS function, which may help to preserve NO homeostasis during the dialysis session. A possible alternative

explanation could be that there exists a difference between the adult and pediatric population in their ability to store and release nitrate and nitrite from intracellular to extracellular compartments. As demonstrated by Piknova and colleagues, skeletal muscle is a significant endogenous reservoir of nitrate, where also nitrite and NO can be formed if needed [50,51]. Further studies are needed to investigate whether differences in intracellular nitrate reservoirs may contribute to the observed variations in NO homeostasis between adult and pediatric ESRD patients undergoing dialysis.

In normal healthy humans, the daily amount of nitrate that originates from NOS-derived NO (as oxidized metabolite) has been estimated to approximately 1 mmol. In a healthy state the kidneys are the main organ for nitrate clearance, but there is less knowledge how this is affected in patients with kidney disease. In our study, the calculated amount of nitrate and nitrite cleared from the body during dialysis were between 0.790 and 1.387 mmol/session and 25.71–36.26 μmol (nitrite) in adults and pediatric patients. This might indicate that the average weekly removal of nitrate by chronic hemodialysis would not be greater than what is normally excreted in healthy individuals. It should be noted, however, that under normal healthy conditions, continuous production and metabolism of NO, followed by renal excretion, is not associated with the significant changes in plasma nitrate, nitrite or cGMP observed in our study population after the dialysis session. Since in ESRD the endogenous NOS-dependent NO generation is reduced [52,53], patients on chronic hemodialysis may rely more on nitrate/nitrite reduction to generate NO than on NOS. In line with this reasoning, it can be assumed that a significant reduction in plasma NOx may have a major effect on NO signaling in patients with ESRD. This speculation is partly supported by the reduction in plasma cGMP levels after dialysis.

Finally, it is worth noting that in a previous study conducted to determine the removal of nitrate and nitrite by hemodialysis in adults with ESRD, blood pressure levels decreased during the dialysis session [12]. The authors of this study speculated that the decrease in blood pressure was due to dialysis-mediated removal of extracellular fluid, that may have offset the effects caused by nitrite removal [12]. In contrast, we found no alterations in post-dialysis blood pressure levels in all our tested groups, even though most of the patients showed a significant reduction in post-dialysis body weight and underwent net ultrafiltration (Supplemental Tables S2 and S3). One explanation is that the lack of change in post-dialysis blood pressure was due to a decreased NO bioavailability, as suggested by the significant reduction in the post-dialysis levels of cGMP (Fig. 4). One caveat is that our analysis was based on the measurement of blood pressure at only two time points, *i.e.*, pre- and post-dialysis. Consequently, we cannot exclude the role of blood pressure variability, a physiological phenomenon characterized by continuous dynamic fluctuations of blood pressure, in the interpretation of our data [54]. It is also important to note that 9 out of 12 critically ill adults with severe acute kidney injury were receiving inotropic support during their dialysis session (data not shown). This may also account for the lack of significant difference between pre- and post-dialysis blood pressure levels this patient group.

4.1. Future perspectives

In view of the well characterized cardiovascular and metabolic effects of nitrate in health and in disease [32], we concur with Bryan and colleagues [12], that an explorative controlled interventional study aiming to replete nitrate levels in ESRD patients and to establish its safety and effectiveness seems to be justified. Based on previous publications [30,32], oral nitrate supplementation, *e.g.*, sodium nitrate tables, may be more targeted and easier to conduct than a study where commercial dialysate fluids are supplemented with nitrate and/or nitrite. This type of clinical trial should initially be performed on adults and ideally, after a pharmacokinetic study designed to evaluate the appropriate dietary nitrate regimen for ESRD patients undergoing hemodialysis has been conducted.

Author disclosure statement

All the authors, apart from Lundberg J.O. and Weitzberg E., declared no competing interests. Lundberg J.O. and Weitzberg E. are co-inventors on patent applications related to the therapeutic use of inorganic nitrate and nitrite. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2020.02.004.

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